Evidence that a Single Peptide–MHC Complex on a Target Cell Can Elicit a Cytolytic T Cell Response

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Summary

Using a chemically homogeneous radiolabeled peptide of high specific activity (^{125}I-QLSPYPFDL, 3.5 × 10^{18} cpm per mole) we show that at a peptide concentration (5 pM) causing half-maximal lysis of target cells by a cytolytic T lymphocyte (CTL) clone that recognizes the peptide in association with L^d, a class I MHC protein, only 3 peptide molecules on average are bound by L^d per target cell. From the distribution of L^d on the target cells, we suggest that a single peptide–MHC complex per target cell can trigger activation of the T cell cytolytic response.

Introduction

In the initial antigen-specific event that leads to T cell activation, antigen-specific receptors on T cells (TCR) react with complexes formed by peptide with major histocompatibility complex (MHC) proteins (pepMHC complexes) on antigen-presenting cells or target cells. As shown in innumerable in vitro assays in which synthetic peptides are incubated with target cells, the intensity of the T cell response depends upon peptide concentration, which, in turn, affects the density of the corresponding pepMHC complexes (epitopes) on target cells. While the number of these complexes per cell (epitope density) appears to be one of the critical determinants of the outcome of T cell–target cell encounters (e.g., Ashton-Rickardt et al., 1994; Sykulev et al., 1994a; Tsomides et al., 1994), measuring them is laborious and only a few values have been reported. In some studies, using radiolabeled peptides and immunoprecipitating the appropriate MHC protein, the minimum epitope density found to trigger antigen-specific T cell proliferation and lymphokine production (Harding and Unanue, 1990; Demotz et al., 1990) or a T cell cytolytic response (Christinck et al., 1991) was around 100–400 complexes per antigen-presenting cell.

Another approach for estimating epitope densities has taken advantage of mutant target cells with a defect in the peptide transporter that results in cell surface class I MHC molecules largely devoid of stably bound peptides (Heemels and Ploegh, 1995). Because of this defect, widely different epitope densities can be established on these cells by incubating them with synthetic peptides at various concentrations, and the average number of pepMHC complexes formed per target cell at steady state can then be estimated from the free peptide concentration; the equilibrium constant for the peptide–MHC reaction; and the total number of accessible (functionally empty) MHC binding sites per cell. Although this approach can be considered to yield only approximate values, it suggested that the epitope densities required for half-maximal cytolytic responses by cytotoxic T lymphocytes (CTLs) varied from several thousand pepMHC complexes per target cell to fewer than ten with different combinations of MHC proteins, peptides, and CTLs (Kageyama et al., 1995). In addition, two independent models for T cell–target cell interactions have proposed that activation of a T cell can be initiated by 3–5 pepMHC complexes (Brower et al., 1994) or by fewer than 10 complexes per target cell (Sykulev et al., 1995).

The low values are of interest because, if correct, they focus attention on a critical question concerning the physiologic activation of T cells: whether T cell triggering involves cross-linking TCR molecules, or whether activation occurs by perturbation of a TCR–multimolecular complex by a single MHC–peptide (Williams and Beyers, 1992). Accordingly, we have sought to determine whether the low values previously suggested by indirect approaches and reliance on models can be verified by measuring directly the number of peptide molecules on antigen-presenting cells under conditions in which these cells elicit a half-maximal cytolytic response by $CD8^+$ T cells. For this purpose, we made use of a chemically homogeneous radioiodinated peptide of exceptionally high specific radioactivity. Prepared with large amounts of carrier-free ^{125}I and purified by high pressure liquid chromatography (HPLC), the peptide had the same specific radioactivity as carrier-free ^{125}I (3.5 × 10^{18} cpm per mole). With this peptide, we found that an average of three pepMHC complexes per target cell could elicit a half-maximal cytolytic T cell response. This finding and the distribution of L^d on the target cells suggest that the cytolytic response of some T cells may be elicited by a target cell that bears a single pepMHC complex.

Results

Peptide Concentration Required for Half-Maximal Cytolysis

CTL clone 2C recognizes pepMHC complexes formed by the class I MHC molecule L^d and one of several naturally processed peptides whose sequences derive from α-ketoglutarate dehydrogenase (Udaka et al., 1993), including p2Ca (LSPFPFDL, Udaka et al., 1992) and QL9 (QLSPFPFDL); the latter is the most potent L^d-sensitizing peptide known for CTL 2C, having an SD_{50} value (concentration required to elicit half-maximal lysis) of 5 pM (Sykulev et al., 1994b), or about 100-fold less than that of peptide p2Ca. To estimate the number of L^d-bound peptide molecules per target cell required to activate 2C cells, we first determined the free peptide concentration that results in half-maximal lysis using

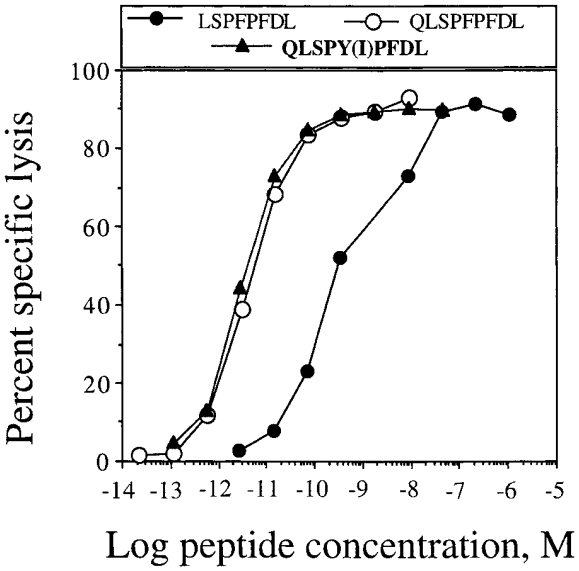

Figure 1. Peptide Concentrations Required for Half-Maximal Lysis

Specific lysis of ^{51}Cr-labeled T2-L^d target cells in the presence of various concentrations of peptides p2Ca (LSPFPFDL), QL9 (QLSPFPFDL), and ^{127}I$_1$-QL9-Y5, the monoiodinated form of QL9-Y5 (QLSPYPFDL).

peptide I$_1$-QL9-Y5 (for sequence see Figure 1), a monoiodinated analog of QL9 available in chemically equivalent nonradioactive (^{127}I) and radioactive (^{125}I) forms. As shown in Figure 1, the SD_{50} value of I$_1$-QL9-Y5, was about equal to that of peptide QL9, or around 5 pM.

Specificity of I$_1$-QL9-Y5 Binding to L^d on T2-L^d Cells

The T2-L^d target cells used in these studies are of human origin and express HLA-A2. Since the sequence of I$_1$-QL9-Y5 happens to fit the A2 consensus motif (it has leucine at positions 2 and 9), and this peptide can bind to A2 as well as L^d (Table 1), it was necessary to include in the reaction mixture, along with I$_1$-QL9-Y5 and T2-L^d cells, a peptide that could saturate the peptide-binding sites of A2 without occupying the peptide-binding sites of L^d. This requirement was met by the influenza virus peptide GILGFVFTL (Bednarek et al., 1991; Morrison et al., 1992), termed GL9. As shown in Table 1, on T2-L^d cells that were incubated with GL9 the level of cell surface A2 increased markedly, but the level of L^d was unchanged. Moreover, GL9 at 1 μM, a concentration in excess over what was required to saturate A2, did not interfere at all with the L^d-dependent lysis of T2-L^d cells by CTL 2C in the presence of peptide p2Ca (data not shown). Thus, by including GL9 at 1 μM in all assays, the binding of I$_1$-QL9-Y5 to A2 could be prevented without interfering with its binding to L^d.

Kinetics of I$_1$-QL9-Y5–L^d Reaction

The rate of binding of I$_1$-QL9-Y5 to L^d on T2-L^d cells was examined to determine whether this peptide–MHC reaction can approach steady-state during standard CTL assay conditions (4 hr). Visual inspection of Figure 2A shows that the number of peptide molecules bound specifically to T2-L^d cells reached steady-state in about 2 hr. When fit to the equation $P_t = \alpha(1 - e^{-t/\tau})$, where P_t is the number of peptide molecules bound specifically per T2-L^d cell at time t, the kinetic data of Figure 2A yielded α, the total number of L^d-bound peptide molecules per cell at steady-state at the free concentration shown (1×10^{-10} M), and also τ, the time constant, i.e., the time required to reach 63% of the steady-state value. This time (τ) was around 50 min. τ becomes longer as the peptide concentration decreases, but it cannot be longer than the reciprocal of the rate constant of dissociation (k_{-1}) for the peptide–L^d reaction (see equation 5a in Sykulev et al., 1994a).

To measure k_{-1}, we made use of an anti-L^d antibody (30-5-7) that reacts specifically with L^d if the binding site is occupied by peptide (Lie et al., 1990): when the peptide dissociates, the resulting empty L^d molecule undergoes rapid denaturation at 37°C. Thus, by loading L^d on T2-L^d cells under conditions where brefeldin A blocked the delivery of newly synthesized L^d to the cell surface, the time course of peptide–L^d dissociation could be monitored by flow cytometry using antibody 30-5-7 (I. V. et al., unpublished data; see Experimental Procedures). From the results shown in Figure 2B, the value of k_{-1} was found to be 3.3×10^{-4} sec^{-1}, yielding an upper limit for τ of about 50 min. Thus, even at the lowest peptide concentration used in this study (5 pM, see below), the I$_1$-QL9-Y5–L^d reaction approached steady-state in about 1 hr.

Table 1. Peptide Binding to HLA-A2 and L^d Proteins on T2-L^d Cells Measured by Enhanced Surface Expression of These MHC Proteins[a]

Peptide concentration (μg/ml)	GILGFVFTL		QLSPY(I$_1$)PFDL	
	HLA-A2[b]	L^d[c]	HLA-A2[b]	L^d[c]
0	136.4	30	136.4	30
0.016	171	26	ND[d]	53.3
0.08	170	25	ND[d]	108
0.4	177.4	26.07	ND[d]	172
2.0	229	25.75	172	190

[a] Mean fluorescence values (linear scale) were determined after 8.5 hr incubation of T2-L^d cells with the indicated peptides at the concentrations shown.

[b] Measured using mouse monoclonal antibody PA2.1 (IgG1, specific for HLA-A2) and FITC-labeled anti-mouse immunoglobulin.

[c] Measured using mouse monoclonal antibody 30-5-7 (IgG2a, specific for the α1 and α2 domains of L^d) and FITC-labeled anti-mouse immunoglobulin.

[d] Not done.

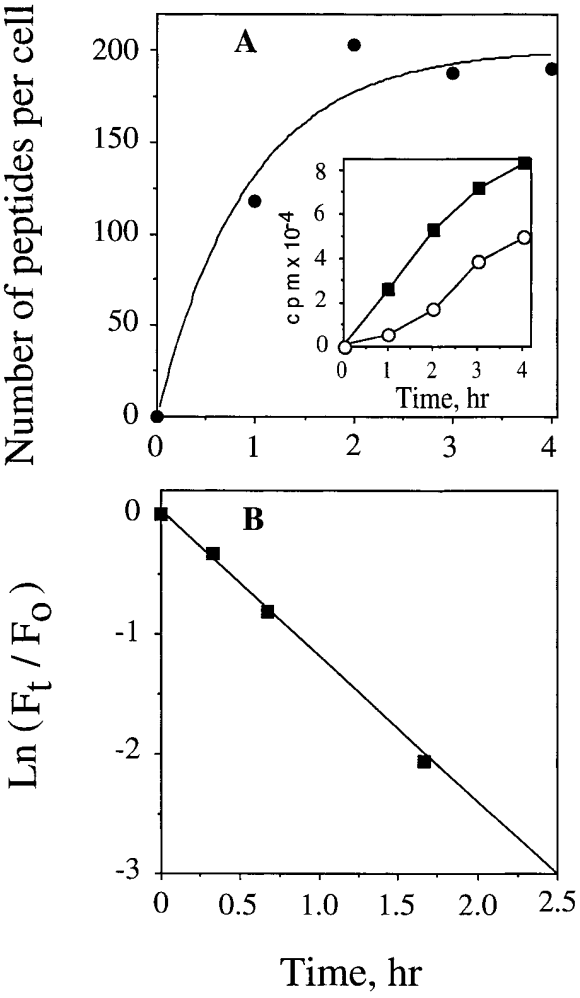

Figure 2. Kinetics of Binding of Monoiodinated Peptide $^{125}I_1$-QL9-Y5 to L^d on T2-L^d Cells at 37°C

(A) The rate of binding of $^{125}I_1$-QL9-Y5 at a free peptide concentration of 10^{-10} M. The increase over time in specific binding of the peptide to L^d is shown by the closed circles. The solid line represents the best fit of the experimental points to the theoretical curve described by the single exponential equation for first order kinetics (see Results for details). The insert shows the change in cell-bound radioactivity (cpm) over time in the presence (open circle) and absence (closed square) of a large (10,000-fold molar) excess of the L^d-binding mouse cytomegalovirus peptide pMCMV. The difference represents peptide specifically bound to L^d.

(B) Rate of dissociation of $^{127}I1$-QL9-Y5 from L^d. Dissociation was monitored by the loss of peptide-stabilized L^d from the surface of brefeldin A–treated T2-L^d cells, measured by the disappearance of FITC fluorescence from antibody-stained cells (see Experimental Procedures). The experimental points (closed square) were fitted to $\ln (F_t/F_o) = -k_{-1} \cdot t$, where F_o and F_t are the mean fluorescence values on a linear scale at time zero and time t, respectively. The slope of a plot of $\ln (F_t/F_o)$ versus t yielded k_{-1}.

Direct Measurement of Epitope Densities

To measure the binding of I_1-QL9-Y5 directly to L^d on target cells, peptide QL9-Y5 was radiolabeled with carrier-free ^{125}I and incubated at 37°C for 3 hr with various concentrations with 5×10^7 T2-L^d cells in a total volume of 5 ml. Free peptide concentrations and the numbers of specifically bound peptide molecules per cell were determined directly from the specific radioactivity of the peptide, 3.5×10^{18} cpm/mol. At three free peptide concentrations, 5×10^{-11}, 2.3×10^{-11}, and 5×10^{-12} M, the directly measured average epitope density values were 50, 40, and 3 pepMHC complexes per cell, respectively.

In view of the implications of these values, it is necessary to consider possible sources of error. One potential error is that ^{125}I–peptide dissociated from L^d when the cells were washed to remove free peptide. This possibility was ruled out by measuring the stability of the peptide–L^d complexes by flow cytometry. The cells were washed at 4°C in about 10 min (see Experimental Procedures), but at this temperature no dissociation could be detected over many hours (data not shown). Another source of error arises from cell counts: we estimate this error to be at most ± 20%. Hence, at the lowest peptide concentration (5×10^{-12} M), the epitope density value in this system was 3 ± 1 pepMHC complexes per target cell. With this average number, the frequencies in the target cell population of cells having 0, 1, 2, 3, 4, and 5 pepMHC complexes per cell would be 0.05, 0.15, 0.22, 0.22, 0.17, and 0.10, respectively, assuming the Poisson distribution to be applicable. In that event, from the sum of these frequencies (0.91) the titration shown in Figure 1 implies that when half the target cells were lysed, most of the lysed cells would have had fewer than five complexes per cell and only about 10%–20% of them would have had more than five per cell.

Is There Selective Lysis of T2-L^d Target Cells Expressing Higher than the Average Number of Peptide–MHC Complexes per Cell?

Alternatively, it could be argued that the distribution of L^d molecules on T2-L^d cells is extremely asymmetric, especially as L^d on these cells is derived from a transfected gene. In that event, only those target cells with L^d levels much above the average might be preferentially lysed. To test this possibility, we compared the distribution of L^d on the initial target cells before they were introduced into a CTL assay with those that survived after 4 hr, after half had been lysed. As shown in Figure 3, the L^d distribution was virtually the same on the initial and surviving target cells. By subtraction, it was clear that the lysed target cells also had a similar distribution.

Discussion

With a high specific–activity radioiodinated peptide it was possible to establish directly that with an average of only three pepMHC complexes per target cell, a $CD8^+$ T cell clone (2C) could be stimulated to make a half-maximal cytolytic response. Although the levels of L^d expressed on the target cells had a broad distribution, it was clear (Figure 3) that there was essentially no preferential lysis of cells having higher than the average number of L^d molecules. Since the probability that a peptide will bind to L^d is proportional to the total number of L^d molecules per cell and to the number of accessible L^d binding sites per cell, the distribution of I_1-QL9-Y5–L^d

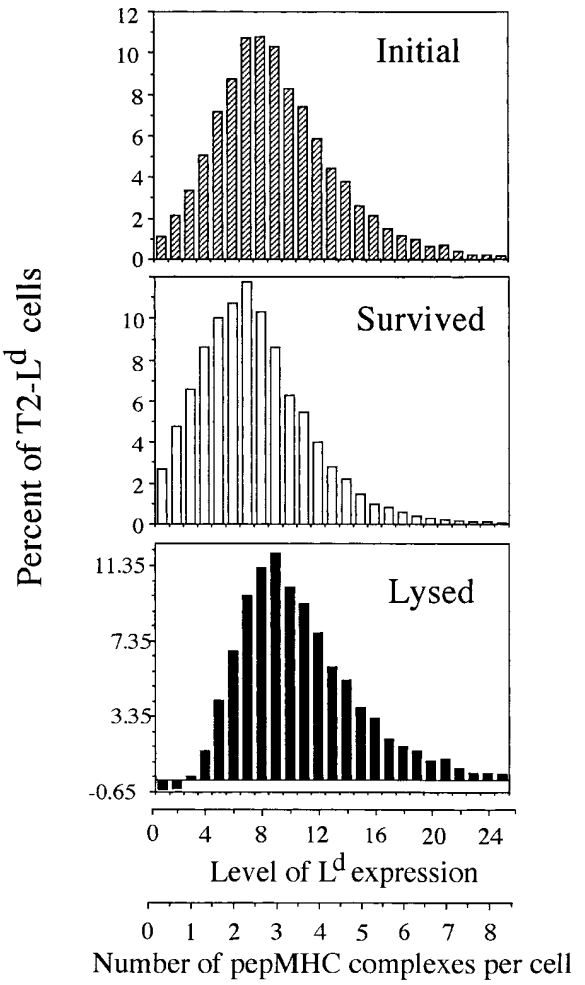

Figure 3. Distribution of L^d and I_1-QL9-Y5–L^d Complexes on T2-L^d Target Cells

Percent of the target cells with different levels of L^d expression is shown for the target cell populations that were incubated for 4 hr at 37°C without the CTL and peptide (the initial population) or with the CTL and the peptide at 5 pM (the survived population). The L^d distribution on the lysed cells was determined by difference (see Experimental Procedures). To determine whether the distribution shown (top) changed over 4 hr, target cells expressing low or high L^d levels were sorted, incubated at 37°C for 4 hr, and examined again (with and without restaining) by flow cytometry. No change in distribution in either subset was seen (data not shown).

complexes on the target cells should match the L^d distribution shown in Figure 3, with the average number of three cognate pepMHC complexes per cell corresponding to the position of the weighted average for total L^d (ninth subset) in this figure. The number of complexes on the lysed cells would thus extend from a low of 1 per cell (Figure 3, third subset at the extreme left) to a high of about 8 (at the extreme right). As seen in the bottom panel of Figure 3, 67% of the lysed target cells had three or more pepMHC complexes per cell, but 33% of the lysed cells had fewer than three and 12% had only 1–2 complexes per cell. Given this extremely low number and the likelihood that pepMHC complexes are randomly distributed over the target cell surface, it is likely that a single complex per target cell can render a cell subject to specific lysis in the 2C system.

Of the directly measured target cell epitope densities required to trigger T cell activity, the value reported here is the lowest by far. Two parameters that determine epitope density requirements are the intrinsic affinity of the TCR–pepMHC reaction and the stability of the TCR–pepMHC bond (bond lifetime). As was recently suggested on the basis of the law of mass action (Sykulev et al., 1995), target cells with epitope densities of 1–10 pepMHC complexes per cell can elicit half-maximal cytolytic responses if the TCR of the CTLs bind these complexes with an intrinsic affinity of about 10^6 M^{-1} or higher. Elsewhere, we show that the TCR on 2C cells does indeed have such an affinity for the I_1-QL9-Y5–L^d complex (Y. S. et al., unpublished data).

How long would a single TCR–pepMHC bond have to persist to elicit a T cell cytolytic response? We previously found that the lifetime of the bond formed by the 2C TCR and the QLSPFPFDL–L^d (QL9–L^d) complex is about 1 min under physiological conditions (intact T cells, 37°C) (Sykulev et al., 1994b). Since peptide I_1-QL9-Y5 behaves very similarly to the QL9 peptide (i.e., it also sensitizes target cells for half-maximal lysis at 5 pM [Figure 1] and the I_1-QL9-Y5–L^d complex is bound by the 2C TCR with high intrinsic affinity), it is likely that under physiological conditions the lifetime of the 2C TCR-I_1-QL9-Y5–L^d bond is also around 1 min. For other systems, having lower intrinsic TCR affinities and forming shorter-lived TCR–pepMHC bonds, higher epitope densities and a larger number of bonds are probably required.

How can the present findings be reconciled with many previous studies suggesting that T cell activation requires multivalent ligands to bring about aggregation or cross-linking of TCR molecules on the T cell surface (e.g., Symer et al., 1992)? In considering the apparent disparity between these findings and ours, we note, first, that the term "activation" covers a multitude of responses. Some are rapid (minutes to hours) and do not require transcription of silent genes, while others are slow (requiring a day or more) and depend upon activation of gene expression. The cytolytic response is relatively fast (typical assay conditions are 4 hr) and is unaffected by treating the T cells with inhibitors of transcription or protein synthesis (e.g., Zychlinsky et al., 1991). That it differs from slow responses such as cell proliferation or cytokine production is evident in split anergy, whereby an antigen-presenting cell lacking costimulatory components was found to render a $CD8^+$ cytolytic T cell clone partially anergic, i.e., the T cells retained their ability to lyse target cells but proved unable subsequently to proliferate (a slow response) in the response to an effective antigen-presenting cell (Otten and Germain, 1991). Studies in this laboratory have also shown that CTLs can be stimulated under conditions in which their cytolytic function is preserved but their proliferative activity is depressed (Dutz et al., 1992).

A single reactive pepMHC on a target cell amounts to a monovalent ligand. In accord with our evidence that such a ligand might trigger a T cell cytolytic response, it has been reported that an increase in T cell intracellular Ca^{2+} concentration, an extremely rapid response, can

be elicited almost as well by the Fab fragment of an anti-CD3 antibody as by the intact bivalent antibody (Oettgen et al., 1985). It has also been shown that monovalent Fab fragments from the same anti-CD3 antibody were able to stimulate interleukin-2 (IL-2) secretion by both syngeneic and allogeneic MHC class II–restricted T cell clones (Tamura and Nariuchi, 1992). Whether a single pepMHC on a target cell can be demonstrated directly to elicit such slow responses is not clear. From the Brower et al. (1994) model, it was deduced that 3–5 pepMHC complexes could stimulate a T cell to produce interferon-γ. However, in that model, class I MHC was adsorbed on plastic to which relatively long peptides were added (10–15 aa in length); whether cytokine production can be elicited by a similar number of pepMHC in a more physiological setting remains to be seen.

Although antigen-induced cross-linking and oligomerization of TCR molecules on the T cell surface are often considered as though they are the same, a distinction between them emerges from the evidence that a single pepMHC complex on a target cell can stimulate a T cell. While a single complex, acting as a monovalent ligand, cannot literally cross-link T cell receptors, it could conceivably bring about their oligomerization. That TCR molecules have a propensity to form dimers is suggested by the recent crystallographic study by Fields et al. (1995), and it can be imagined that monovalent pepMHC ligation of a TCR could enhance this tendency, perhaps by inducing a conformational change in the TCR (e.g., Rojo and Janeway, 1988). In an analogous situation, conformational changes of immunoglobulin resulting from antigen binding have been demonstrated (e.g., Stanfield et al., 1993).

While a single pepMHC can react with only a single TCR at any instant, over time it can engage many of them, reacting repetitively with the same TCR molecule or serially with many different TCR molecules as recently emphasized by Valitutti et al. (1995). The number of engagements associated with a given T cell response depends, in part, on the lifetime of the TCR–pepMHC bond. The few dissociation rate constants reported so far indicate that the lifetimes of these bonds range from about 1–100 s (Sykulev et al., 1994a, 1994b; Matsui et al., 1994; Corr et al., 1994), but how long the bonds have to persist in order to elicit particular responses remains to be determined.

Experimental Procedures

Cells

The $CD8^+$ cytolytic T cell clone 2C was maintained as described (Kranz et al., 1984). The human hybridoma T2 transfected with the L^d gene (Alexander et al., 1989) was cultured in K medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol) in the presence of 320 μg/ml G418.

Peptides

Except for the influenza virus matrix peptide GILGFVFTL, which was provided by Drs. M. Bouvier and D. Wiley (Harvard University, Cambridge, Massachusetts) (Bouvier and Wiley, 1994), all other peptides were synthesized by solid phase t-Boc chemistry in the Massachusetts Institute of Technology Biopolymers Laboratory and purified by reverse phase HPLC. The monoiodinated nonradioactive form of peptide QLSPYPFDL, termed $^{127}I_1$-QL9-Y5, was prepared in the same way, using t-Boc-protected monoiodinated tyrosine (Peninsula, Incorporated, Belmont, California). Because the N-terminal glutamine rearranges to cyclized pyrrolidone carboxylate, the peptide exists in two forms (separable by HPLC). Both were recognized by 2C cells in association with L^d. The preparations used here were predominantly in cyclized form. All peptide concentrations were measured by quantitative amino acid analyses.

Stoichiometric Iodination of Peptide QLSPYPFDL (QL9-Y5) with ^{125}I

The monoiodinated radioactive form of peptide QL9-Y5 was prepared by iodination with carrier-free $Na^{125}I$ (Du Pont NEN) as described (Tsomides and Eisen, 1993; Schumacher and Tsomides, 1995). In brief, in a typical reaction, 200 μg of purified peptide QL9-Y5 was reacted with 30 mCi $Na^{125}I$ using two Iodo-Beads (Pierce) for 30 min at pH 6.0. After unbound iodide was removed from the peptide mixture using a Sep-Pak C18 cartridge (Waters) in a glove box, the labeled peptide products and unreacted QL9-Y5 were resolved from one another on a 4.6 $\times$ 250 mm reverse phase C18 HPLC column (Vydac) with a 1%/min acetonitrile gradient, using online radioisotope detection (Beckman model 170). Individual radiolabeled products were dried, resuspended in H_2O, and aliquots were counted in a γ counter (Packard) with a known counting efficiency of 75% to determine the specific radioactivities of the peptides. The specific radioactivity of monoiodinated $^{125}I_1$-QL9-Y5 was the same as that of carrier-free ^{125}I (3.5×10^{18} cpm/mol). The composition of this peptide was further confirmed by Edman degradation after HPLC purification (Tsomides and Eisen, 1993).

Antibodies

Mouse monoclonal antibodies PA2.1 (IgG1, anti-HLA-A2; Brodsky et al., 1979) and 30-5-7 (IgG2a, anti-L^d; Ozato et al., 1980; Lie et al., 1990) were isolated from supernatants of hybridoma cell cultures using protein A affinity chromatography.

Cytolytic Assay

Peptides (50 μl) at various concentrations in phosphate-buffered saline (PBS) were added to 5×10^3 ^{51}Cr-labeled target cells (T2-L^d) and 1.5–2.5 $\times$ 10^4 CTL (2C) in 150 μl of K medium in round-bottomed wells of microtiter plates. After brief centrifugation (300 $\times$ g for 5 min), the plates were incubated in a CO_2 incubator for 4 hr at 37°C. Percent specific lysis was calculated from the average of duplicates as 100 $\times$ ([^{51}Cr experimentally released $-$ spontaneous release] / [total release in 0.1% NP40 $-$ spontaneous release]).

Flow Cytometry

To measure peptide-dependent stabilization of A2 and L^d molecules on T2-L^d cells, the cells were incubated with various peptide concentrations at 37°C for 6–12 hr. Expression of cell surface A2 and L^d was detected with antibodies PA2.1 and 30-5-7, respectively, followed by staining the cell-bound antibodies with fluorescein isothiocyanate (FITC)–labeled $F(ab')_2$ fragments of goat antibodies to mouse immunoglobulins. The levels of expression of the MHC proteins were measured by mean fluorescence on a linear scale.

Kinetic Analysis of the Reaction Between I_1-QL9-Y5 and L^d on T2-L^d Cells

Sixteen tubes, each containing 5×10^7 cells in 5 ml K medium, were incubated with influenza virus matrix peptide GL9 at 1–2 μM for 1 hr at 37°C. I_1-QL9-Y5 was then introduced into all tubes at 10^{-10} M. To correct for nonspecific binding of the ^{125}I–peptide to the cells, peptide YPHFMPTNL (pMCMV, Reddehase et al., 1989) was added to half the tubes at a final concentration of 1–2 $\times$ 10^{-6} M; this concentration greatly exceeds what is required to saturate the L^d binding sites, since the equilibrium binding constant for the pMCMV–L^d reaction is about 2×10^9 M^{-1} (Sykulev et al., 1994a). The tubes were slowly rotated on a stirring wheel at 37°C and after each hour duplicate tubes containing the labeled peptide only (to measure total binding) and duplicate tubes containing both the labeled peptide and the L^d-blocking unlabeled peptide were centrifuged at 4°C. After removing supernatants, which were counted to measure the free concentration of ^{125}I–peptide, the cell pellets were

washed twice with ice-cold K medium, resuspended in 2–3 ml of ice-cold K medium, and layered on 2 ml of silicone-paraffin oil mixture (see above) and centrifuged at 4°C. After freezing the centrifuge tubes, the tips were cut off to measure cell-bound ^{125}I–peptide. Dissociation of the labeled cell-bound peptide during manipulations at 4°C was shown to be negligible (I. V. et al., unpublished data).

To determine the rate constant of dissociation (k_{-1}) of I_1-QL9-Y5 from L^d, 5×10^6 T2-L^d cells were preincubated in a total volume of 200 μl with 5 μM brefeldin A for 40 min at 37°C to block delivery of newly produced MHC molecules to the cell surface. Peptide $^{127}I_1$-QL9-Y5 was then added at a final concentration of 4×10^{-4} M and incubation was continued at 37°C. After the cell suspension was subjected to rapid centrifugation, the supernatant was promptly removed and the cells were washed twice with ice-cold K medium and resuspended in K medium that was preheated to 37°C and contained 5 μM brefeldin A. An aliquot of the cell suspension was taken immediately (time zero) and placed on ice. The remaining cells were kept at 37°C and samples were removed every 15–20 min, placed on ice, and stained with 30-5-7 anti-L^d antibody as described above, to determine their mean L^d expression by flow cytometry. The results were analysed as described in the legend to Figure 2.

Direct Measurement of I_1-QL9-Y5–L^d Complexes on T2-L^d Cells

The number of I_1-QL9-Y5–L^d complexes on T2-L^d cells was measured directly with cells that were incubated at 37°C for 3 hr (Figure 2, see equilibrium time) with $^{125}I_1$-QL9-Y5 at three different concentrations: 5×10^{-11}, 2.3×10^{-11}, and 5×10^{-12} M. We added peptide $^{125}I_1$-QL9-Y5 to four tubes, each prepared with 5×10^7 cells as above, to achieve one of the indicated concentrations in the presence or absence of unlabeled pMCMV at a concentration that greatly exceeds what was required to saturate L^d. All tubes contained peptide GL9 at 1–2 μM to saturate peptide binding sites on A2 (which is present on T2-L^d cells). After 3 hr, cell-bound and unbound ^{125}I–peptide were separated and measured as above. The number of L^d-bound peptide molecules per cell was determined from the difference between total cell-bound cpm measured in the absence and presence of the L^d-saturating concentration of peptide pMCMV; the specific radioactivity of $^{125}I_1$-QL9-Y5 (3.5×10^{18} cpm per mole); and the number of cells in the sample. The amount of specifically cell-bound ^{125}I–peptide was the same when the pMCMV peptide was incubated with the cells for 1 hr at 37°C before the addition of $^{125}I_1$-QL9-Y5 and when the ^{125}I–peptide and pMCMV were added at the same time.

The Distribution of L^d Molecules on T2-L^d Target Cells

T2-L^d target cells were labeled with the PKH26 dye according to the protocol of the manufacturer (Zynaxis Cell Science, Incorporated, Malvern, Pennsylvania), and they were then labeled in the standard way with ^{51}Cr. Double-labeled target cells (5×10^3) were incubated with various concentrations of $^{127}I_1$-QL9-Y5 and 1.5×10^4 2C cells in a total volume of 200 μl in round-bottomed 96-well plates for 4 hr at 37°C. Percent specific lysis was determined by counting ^{51}Cr in the supernatants as decribed above. The presence of PKH26 on the target cells had no effect on their specific lysis by CTL 2C. In parallel, T2-L^d targets tagged with PKH26 and not ^{51}Cr-labeled were incubated under precisely the same conditions at the peptide concentrations required for 20%–80% of maximal lysis, i.e., 1×10^{-12} to 3×10^{-11} M, using 24 identical wells for each of the peptide concentrations tested. Two control 24-well sets of the target cells were also examined at the same time: the first set contained only target cells and are referred to below as the initial cells; the second set contained the target cells and CTL 2C but no added peptide. At the end of the incubation period (4 hr), cells from each 24-well set were pooled, immediately chilled on ice, and stained with 30-5-7 anti-L^d antibody as described above. After adding propidium iodide (PI) to detect dead cells, all samples were analysed by flow cytometry on the FACStar Plus flow cytometer. About 90% of the initial target cells were PI^-, whether or not they had been incubated alone or with CTL 2C in the absence of the peptide. However, in the presence of the peptide the fraction of surviving target cells ($PKH26^+PI^-$) was reduced and the decrease was greater as the peptide concentration increased.

The levels of L^d on the intitial and surviving T2-L^d target cell populations ($PKH26^+PI^-$) were compared by flow cytometry by measuring FITC fluorescence intensity of the antibody-stained cells on a linear scale. The fluorescence of these cells fell within channels 20 and 520, and this region was divided into 25 equal intervals. Numbered from 1–25, the intervals provided a relative measure of the cell surface level of L^d. The distributions of L^d on both the intitial and surviving target cell populations were displayed by plotting the percent of all $PKH26^+PI^-FITC^+$ cells for a given population in each of the numbered (n) intervals. To determine the L^d distribution on the lysed target cells, we calculated the percent of lysed cells in each interval (P_n^k) from:

$$P_n^k = \frac{P_n^{int} - (1 - f_k)\, P_n^s}{f_k}$$

where P_n^{int} and P_n^s are the percentages of initial and survived target cells, respectively, in the n^{th} interval, and f_k is the fraction of specifically lysed cells determined from released ^{51}Cr. Values of P_n^k were plotted as a function of the interval number n. Relative weighted average values (means) of L^d expression ($\bar{n}$) in all three populations of target cells (initial, survived, and lysed) were calculated as:

$$\bar{n} = \frac{\sum_{n=1}^{25} P_n \bullet n}{100}$$

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